United States Department of Agriculture
Food Safety and Inspection Service, Office of Public Health Science

SOP No: CLG-TST2.00		Page 1 of 21
Title: Confirmation of Thyreostats by HPLC/MS/MS		
Revision 00	Replaces: NA	Effective: 12/03/04

Contents

A. INTRODUCTION

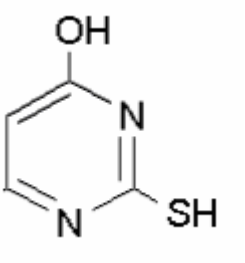
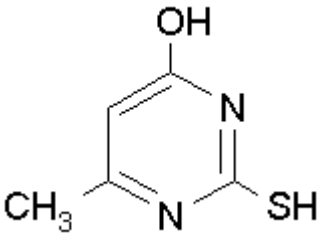
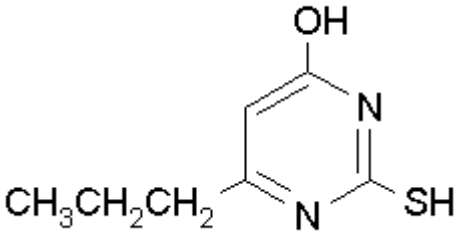
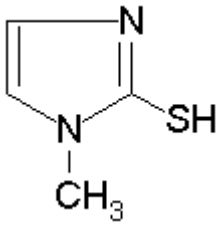
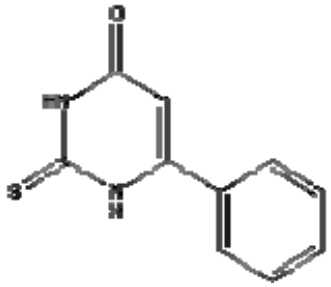
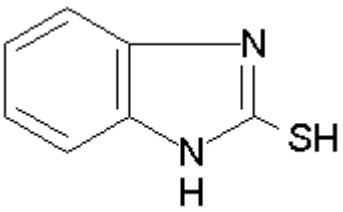
1. Theory

Thyreostat residues are extracted with acetonitrile from muscle homogenate. The extract is partially cleaned by passing through the silica gel column, and then analyzed by HPLC-MS/MS for confirmation. Confirmation is based on comparison of sample MS/MS spectral data with that of a fortified tissue standard or external standard.

2. Applicability

This method will confirm thyreostats (2-thiouracil, 6-methyl-2-thiouracil, 6-propyl-2-thiouracil, 6-phenyl-2-thiouracil, 2-mercapto-1-methylimidazole, and 2-mercaptobenzimidazole) in porcine, equine and bovine muscle at ≥ 25 ppb.

3. Structures

THYREOSTATS	
	
2-thiouracil (TU) (MW = 128)	6-methyl-2-thiouracil (MTU) (MW = 142)
	
6-propyl-2-thiouracil (PrTU) (MW = 170)	2-mercapto-1-methylimidazole or Tapazole (TAP) (MW = 114)
	
6-phenyl-2-thiouracil (PhTU) (MW = 204)	2-mercaptobenzimidazole (MBI) (MW = 150)

B. EQUIPMENT

1. Apparatus

   Note: Equivalent apparatus and instrumentation may be substituted for the following:

   a. Robot Coupe® Processor - Robot Coupe U.S.A. Inc.

   b. Centrifuge tubes - 50 mL, polypropylene tube, Falcon Cat. No. 352070, Becton Dickinson Labware.

   c. Pipettors - 5 -100 µL, 100 -1000 µL, Rainin EDP variable volume micropipettes.

   d. Top-loading balance - PM 300, Mettler.

   e. Analytical balance - Leco-250, Leco Corp.

   f. Conical reaction vials - 5mL, Kontes Microflex, #749000-0005.

   g. Microfilterfuge tubes - 45 µm Nylon 66, Cat. No. 7016-22, Rainin.

   h. Evaporator - N-Evap, Organomation Associates.

   i. Nitrogen source - Whatman N2-2010-(75-86).

   j. Volumetric flasks - 1 L, 20, 10 and 1 mL.

   k. Graduated cylinder - 1 L, 500 mL, 10 mL.

   l. HPLC solvent filtering apparatus - with 0.45 µm filter.

   m. Freezer - capable of attaining < - 20 ºC.

   n. Pipettes - 0.5 mL, 1 mL and 2 mL.

   o. Centrifuges - Sorvall T6000B and VWR Model V.

   p. Vortex mixer - Genie 2, Fisher Scientific.

   q. Sonicating water bath - Aquasonic, Cat. No. 150T, VWR.

   r. Glass centrifuge tubes - 15 mL, Cat. No. 8084, Pyrex.

   s. Pasteur pipettes - disposable glass, 9 in. long.

   t. HPLC vials and inserts.

   u. SPE column - Silica Gel, Bond Elute, 0.5 g, 10 mL, Cat. No. 1211-3036, Varian.

2. Instrumentation

   a. Micromass Quattro Micro equipped with electrospray LC interface coupled to a Waters 2695 HPLC and autosampler.

   b. LC column - Phenomenex Prodigy 3 µ ODS(3) 100A 4.6 mm x 150 mm.

   c. Guard column - Phenomenex ODS 4 mm x 3 mm.

C. REAGENTS AND SOLUTIONS

Note: Equivalent reagents and solutions may be substituted.

1. Reagents
   a. Acetonitrile (CH_3CN) - Cat. No. 015-4, Burdick & Johnson.
   b. Methylene chloride (CH_2Cl_2) - Cat. No. 9264-03, J. T. Baker.
   c. Methanol (MeOH) - Cat No. 230-4, Burdick & Johnson.
   d. Sodium sulfate - Anhydrous, Cat. No. S421-1, Fisher Scientific.
   e. Formic Acid (HCO_2H) - Cat. No. 06440, Fluka.
   f. Water - HPLC grade.

2. Solutions

   Note: Unless otherwise noted, solutions may be stored at room temperature.

   a. 25% MeOH/CH_2Cl_2 (v/v):

      Mix 1 part MeOH with 3 parts CH_2Cl_2.

   b. HPLC mobile phases:

      A = 0.1% HCO_2H - Mix 1 mL HCO_2H with 1L HPLC water.

      B = 0.1% HCO_2H in 1:1 CH_3CN:MeOH - Mix 1 mL HCO_2H with 1 L 1:1 CH_3CN:MeOH

D. STANDARDS

1. Source

   Note: Equivalent sources for the standards can be used.

   2-Thiouracil - Cat. No. 301507, Aldrich.

   6-Methyl-2-thiouracil - Cat. No. 69400, Fluka.

   6-Propyl-2-thiouracil - Cat. No. 82460, Fluka.

   6-Phenyl-2-thiouracil - Cat. No. P3252, Sigma.

   2-Mercapto-1-methylimidazole - Cat. No. 301507, Aldrich.

   2-Mercaptobenzimidazole - Cat. No. M3205, Aldrich.

2. Preparation

   a. Stock Standard Solutions (1 mg/mL):

      Accurately weigh 20.0 ± 1 mg of each of the above standards into separate scintillation vials. Dissolve in 20 mL methanol. Store at < -10 °C.

b. Working Standard Solution (3 µg/mL):

Pipet 30 µL each stock standard to a 10 mL volumetric flask and bring to volume with methanol. Store at room temperature.

c. LC Standard Solution (25 ng/mL):

Pipet 10 µL working standard into LC vial and dilute with 400 µL methanol and 800 µL 0.1% formic acid. Prepare as needed and store at room temperature.

d. Storage and Stability: The stock standard solution is stable for 6 months when stored in a freezer ≤ - 10 °C.

E. SAMPLE PREPARATION

After removing excess fat from sample, homogenize with a food processor, transfer into plastic bags and store in a freezer at ≤ - 10 °C. Let the sample thaw prior to analysis.

F. ANALYTICAL PROCEDURE

1. Extraction

a. Weigh 5 g homogenized tissue into 50 mL polypropylene centrifuge tube.

Note: At this time, weigh two 5 g portions of blank muscle tissue into 50 mL polypropylene centrifuge tubes. Use the first tube as a blank and fortify the second tube as a recovery by adding 42 µL of working standard (D.2.b) for a 25 ppb recovery.

b. Add 10 mL acetonitrile and cap.

c. Shake vigorously and vortex at least 1 minute until sample is dispersed.

d. Centrifuge at about 2500 rpm about 5 minutes.

e. Remove about 5 mL solution and place in 15 mL glass centrifuge tube.

f. Evaporate on N-Evap to dryness at ≤ 60 °C.

g. Add 0.5 mL methylene chloride to sample tube, cap and vortex briefly.

h. Sonicate at least 5 minutes.

2. SPE Column Cleanup

Note: Do not let the SPE column to go to dryness during steps 2.b-f below. Also for steps 2.b-f allow liquid level to drain to top of column before adding next volume of liquid.

a. Add 1 g sodium sulfate to a silica gel SPE column, and position over waste container.

b. Wash column with 2 mL methylene chloride.

c. Add sample extract (1.h) to column.

d. Add 0.5 mL methylene chloride to sample tube, vortex and sonicate briefly, and add to column.

e. Add 2 mL 25% methanol/methylene chloride to sample tube and sonicate at least 1 minute.

f. Wash column with 2 mL methylene chloride.

g. Remove waste container and position a 5 mL reaction vial under column.

h. Elute column with 25% methanol/methylene chloride from (e). Allow eluate to fully drain into tube.

i. Evaporate to dryness on N-Evap at ≤ 60 °C.

j. Add 200 µL methanol and 400 µL 0.1% formic acid.

k. Cap and vortex briefly.

l. Add to 0.45 µm filterfuge tubes.

m. Centrifuge at about 8000 rpm until sufficient volume of filtrate has been collected for HPLC analysis.

n. Place sample in LC injection vial.

2. Instrument Operating Parameters - LC System

Note: The instrument parameters listed here are examples of one set of suggested optimization parameters. Others may yield equivalent results. The analyst should optimize parameters for the instrument used.

a. Set initial composition of mobile phase A to 93% and B to 7% at a flow rate of 0.5 mL/min. Allow system to equilibrate.

b. Injection volume: 20 µL

c. Elution gradient:

Time (min)	Flow Rate (mL/min)	Mobile phase A (%)	Mobile phase B (%)
0	0.5	93	7
6	0.5	93	7
20	0.5	20	80
23	0.5	20	80
25	0.5	93	7
28	0.5	93	7

3. Mass Spectrometer Setup:

   Program the mass spectrometer to collect the product ions.

4. Instrumental Settings

   Note: Table contains recommended values. Instrumental settings may be adjusted, if necessary, to optimize performance.

   Typical LC/MS system setting:

Polarity	ES+
Source Temperature	120 ºC

   Note: See Section K. 1. for additional settings.

5. Injection Sequence

   a. Inject external standard mixture and recovery. Verify that all monitored product ions are present in the external standard.

   b. Inject the recovery and blank. Verify the absence of analyte carry over in the blank. If significant carry over is detected, inject solvent/ blank until reduced to acceptable level.

   c. Inject sample extract(s). Include additional washes and standards in the run as often as necessary to ensure proper identification of sample analytes.

   d. Reinject standard or recovery at the end of the run to verify instrument response.

6. Sample Chromatograms

   See Section K.2, Sample Chromatograms

G. DETECTION AND CONFIRMATION

1. For each injection:

   a. Plot ion chromatograms for each product ion monitored.

   b. Determine retention times and abundances for all product ions.

   c. Calculate the ratios of product ions specified below for confirming testing.

Analyte	Product Ions	Parent mass	Retention Time examples
2-Thiouracil (TU)	84 & 112	129	5.32
2-Mercapto-1-methylimidazole (TAP)	88 & 56	115	6.93
6-Methyl-2-thiouracil (MTU)	126 & 84	143	7.61
6-Propyl-2-thiouracil (PrTU)	154 & 112	171	16.42
2-mercaptobenzimidazole (MBI)	118 & 93	151	17.09
6-Phenyl-2-thiouracil (PhTU)	188 & 146	205	18.63

2. Confirmation Criteria

Confirmation of thyreostat residues in a sample extract requires that the following criteria be met:

a. Retention time of the product ion peaks in the sample chromatograms must match that found in the external standard or recovery within ± 4%.

b. At least 2 product ion peaks characteristic of the analyte are present with a signal to noise ratio of greater than 3.

c. If two product ions are monitored, the presence of one sample ion ratio match that calculated for the external or recovery within a ± 10% arithmetic difference.

d. The blank does not have confirmable analyte(s).

H. SAFETY INFORMATION AND PRECAUTIONS

1. Required Protective Equipment - Protective clothing, eyewear, gloves, and a hood where applicable.

2. Hazards

Procedure Step	*Hazard*	*Recommended Safe Procedures*
TU	May be harmful by inhalation, ingestion/skin absorption. Target organs Liver and Thyroid. May cause irritation to skin, mucus membranes and eyes.	Use a hood and wear protective clothing and gloves when handling standards.

MBI	Teratogen. May be harmful by inhalation, in contact with skin and if swallowed. Possible risk of impaired fertility and possible fetus risk.	See above
TAP	Possible Teratogen. May be harmful by inhalation, in contact with skin and if swallowed. Possible risk of impaired fertility and possible fetus risk.	See above
PhTU	May be harmful by inhalation, ingestion and skin absorption. Irritation to mucus membranes and upper respiratory tract.	See above
PrTU	Possible Carcinogen. May be harmful by inhalation, ingestion and skin absorption. May cause skin irritation. Irritation to mucus membranes and upper respiratory tract.	See above
MTU	Possible Carcinogen. May be harmful by inhalation, ingestion, and skin absorption. May cause skin irritation. Irritation to mucus membranes and upper respiratory tract.	See above
Acetonitrile/methanol	Flammable, poisonous liquid	Wear protective clothing and gloves when handling acetonitrile and methanol.

Formic acid	Dangerously caustic to skin. Chronic absorption has been reported to cause albuminuria and hematuria.	Wear protective clothing and gloves when handling formic acid.

3. Disposal Procedures

Procedure Step	*Hazard*	*Recommended Safe Procedures*
Thyreostat standards as stated in above table.	See above.	Collect waste in a sealed container and store in a cool, well ventilated, flammable liquid storage area/cabinet for disposal in accordance with local, state, and Federal regulations.
Acetonitrile/methanol	See above.	See above
Formic acid	See above.	See above

I. QUALITY ASSURANCE PLAN

1. Performance Standard
   a. No false positives from blank tissues.
   b. No false negatives at ≥ 25 ppb level fortification.

2. Critical Control Points and Specifications

	Record	*Acceptable Control*
a.	F.2.b-f. Silica gel column wash	Column must not go to dryness

3. Readiness To Perform (FSIS Training Plan)
   a. Familiarization
      i. Phase I: Standards. Inject a mixed standard solution containing all six thyreostats at concentration equivalent to 25 ppb in sample extracts. Repeat analysis on three different days.
      ii. Phase II: Analyst fortified samples. Analyze one blank beef muscle tissue and beef muscle tissue fortified with 25 ppb mixed standards. Repeat the analyses two more days using blank pork muscle for day 2 and blank horse muscle for day 3.

          NOTE: Phase I and Phase II may be performed concurrently.

      iii. Phase III: Check samples for analyst accreditation.
          (a) 6 samples fortified at 25 ppb level of each analyte. Any combination of species may be used, and set must include 1 blank.
          (b) Report analytical findings to Supervisor and QAM.
          (c) Letter from QAM is required to commence official analysis.

   b. Acceptability criteria.

      Refer to I. 1.

4. Intralaboratory Check Samples
   a. System, minimum contents.
      i. Frequency: One per week per analyst when samples are analyzed.
      ii. Records are to be maintained.
   b. Acceptability criteria.

Refer to I. 1.

If unacceptable values are obtained, then:

i. Stop all official analyses by that analyst.

ii. Take corrective action.

5. Sample Acceptability and Stability

a. Matrices: Bovine, porcine or equine muscles.

b. Sample receipt, minimum weight: approximately 500 g.

c. Condition upon receipt: chilled or frozen.

d. Sample storage:

i. Condition: frozen (≤ -10 ºC) for blended/homogenized samples.

6. Sample Set

Each set must include the following:

a. Blank muscle (negative control).

b. Muscle recovery (positive control).

c. Samples.

7. Sensitivity

a. Minimum proficiency level (MPL): 25 ppb

J. WORKSHEET

Thyreostat Analysis by LC-MS-MS

Analyst(s):_____ Started:_____ Completed:_____ Reviewer:_____	Method: TST2.___
Equipment: Balance:_____ CH_2Cl_2:_____ N-Evap:_____ CH_3CN:_____ Pipettes:_____ Na_2SO_4:_____	LC-MS-MS: ____ ESTD:_____ Fortification Std:_____ Mobile Phase A:_____ Mobile Phase B:_____ MeOH/CH_2Cl_2:_____

Sample ID	Sample Type	Sample Weight(g)	Comments
Neg. Control			
Pos. Control			

K. APPENDIX

1. Additional Instrument settings:

Calibration static	2
Capillary voltage (kV)	4
Cone voltage	35
Extractor voltage	2
RF Lens voltage	0.20
Desolvation Temperature	400 ºC
Cone Gas Flow (L/H)	91
Desolvation Gas Flow	512
LM 1 Resolution	15.0
HM 1 Resolution	15.0
Ion Energy	10.4
Entrance	5 - 16
Collision	25 - 21
Exit	1 - 22
LM 2 Resolution	15.0
HM 2 Resolution	15.0
Ion Energy	22.0
Multiplier voltage	22.0
Gas Cell Pirani Pressure (mbar)	5.98e - 3

2. Mass Spectra

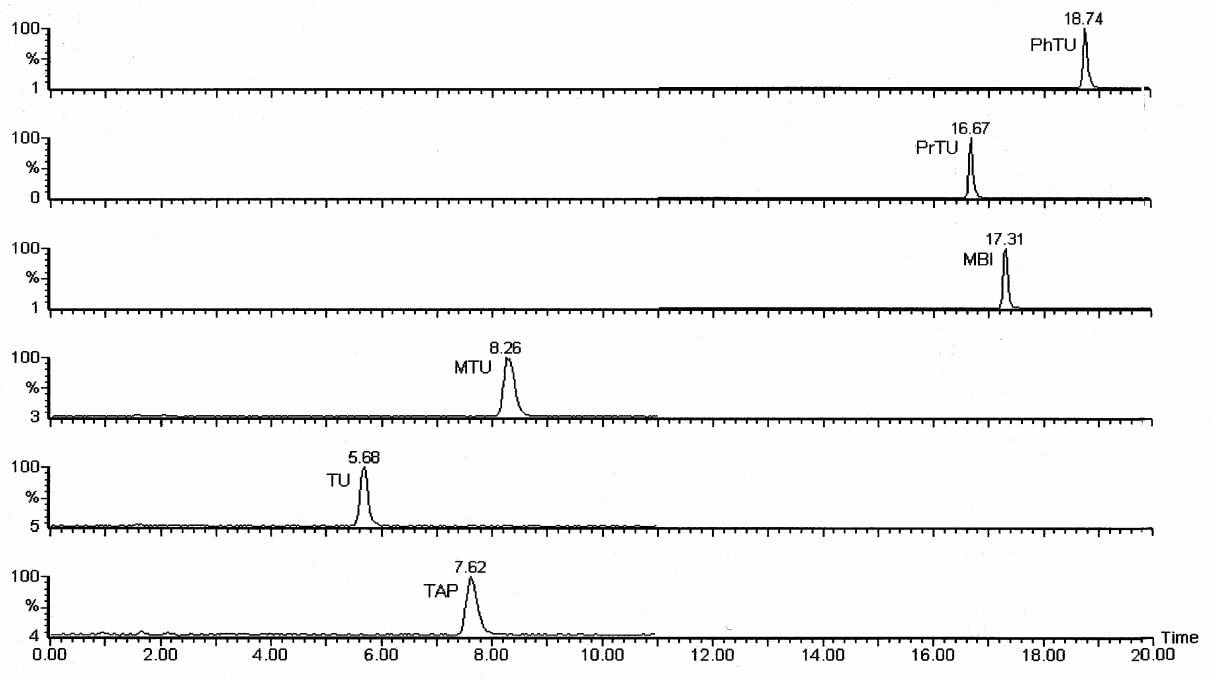


Figure 1. Spectra of 25 ppb Thyreostat standards

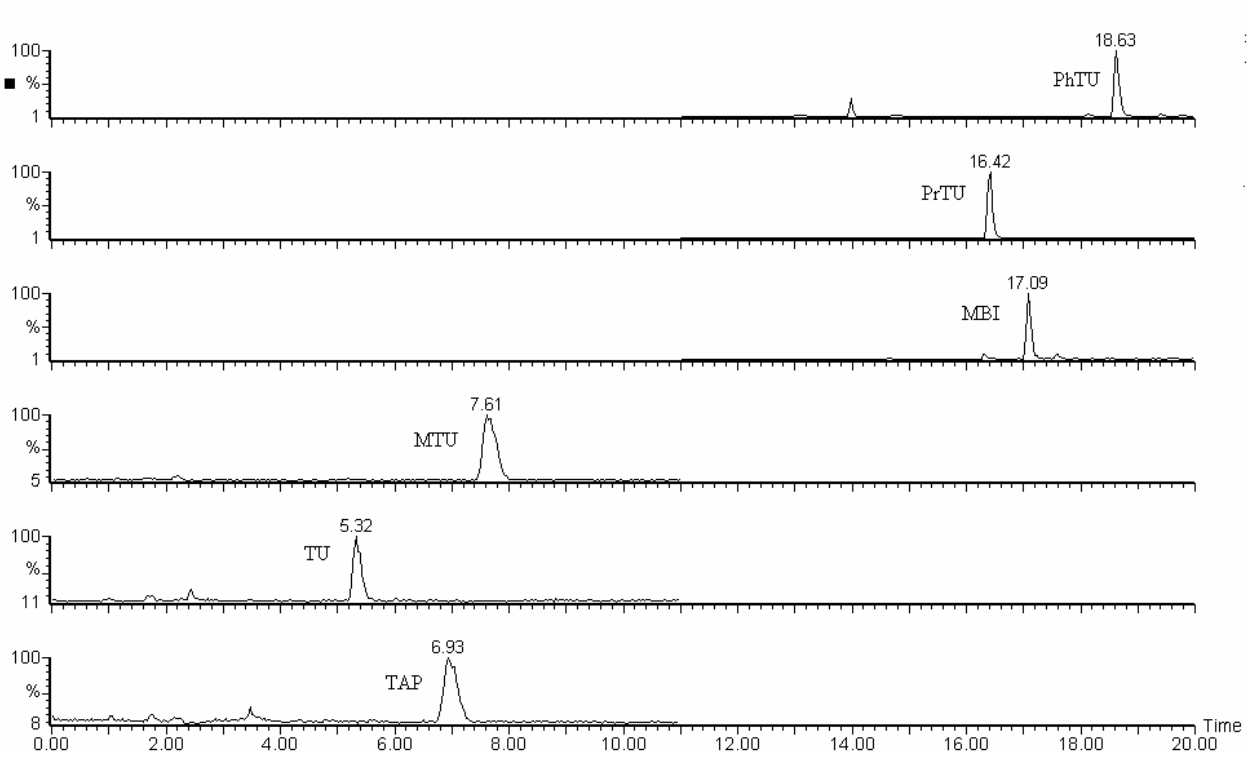


Figure 2. Spectra of 25 ppb Thyreostats Recovery

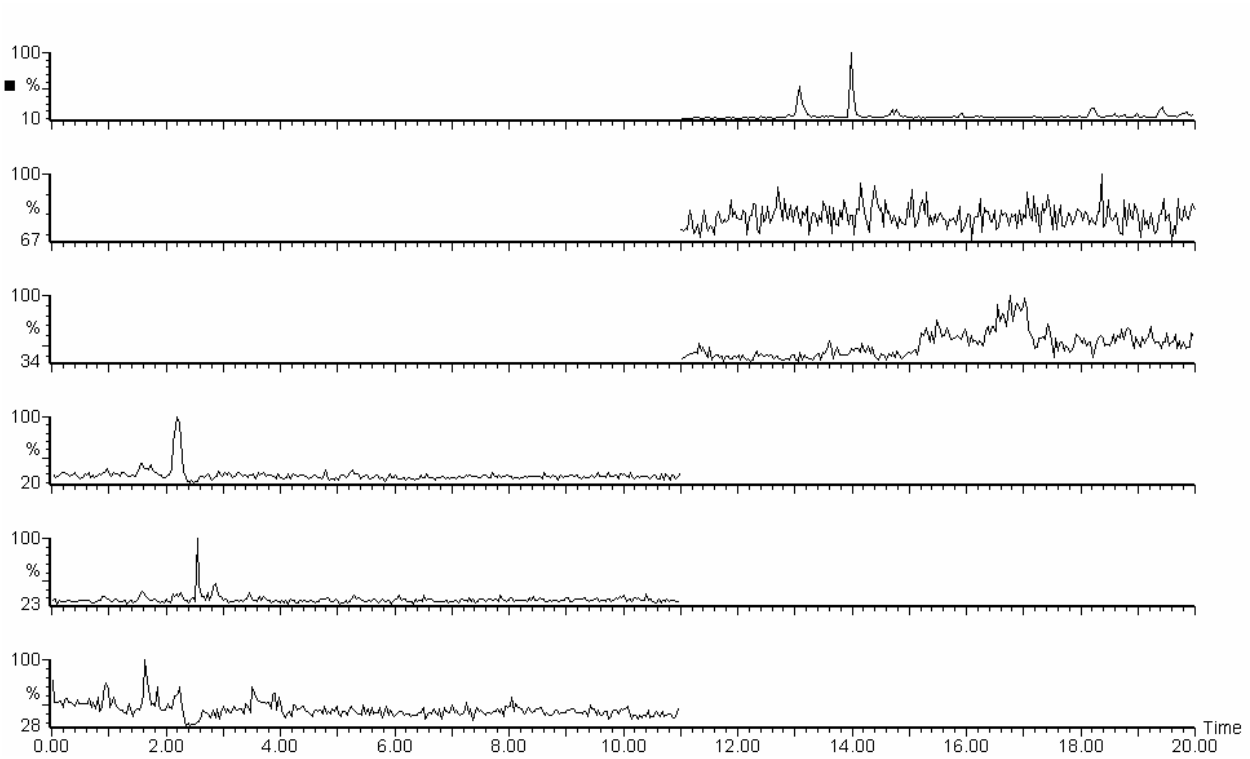
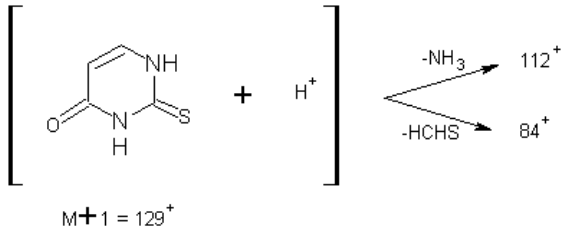
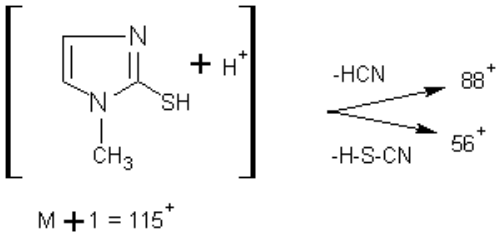
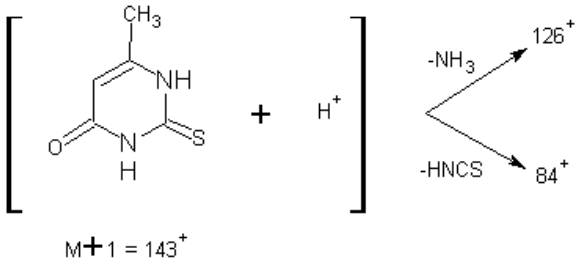
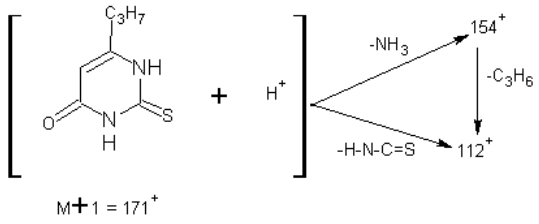
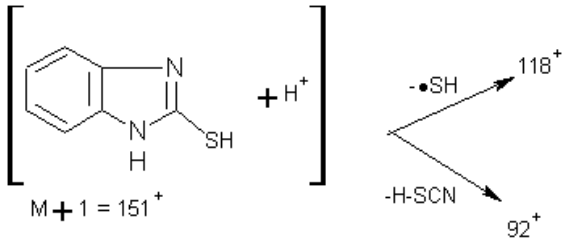
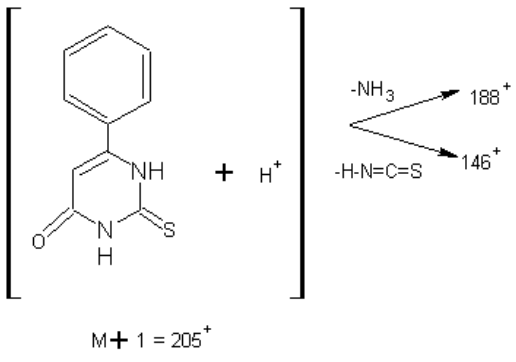


Figure 3. Spectra of Blank Muscle

3. Possible Fragmentation Patterns of Thyreostats Product Ions

2-thiouracil (TU) (MW = 128)	2-mercapto-1-methylimidazole or tapazole (TAP) (MW = 114)
	
6-methyl-2-thiouracil (MTU) (MW = 142)	**6-propyl-2-thiouracil (PrTU) (MW = 170)**
	
2-mercaptobenzimidazole (MBI) (MW = 150)	**6-phenyl-2-thiouracil (PhTU) (MW = 204)**
	

3. Reference

Steven J. Lehotay, Alan R. Lightfield, Nichelangelo Anastassiades, and David J. Smith. "Simultaneous Analysis of Beta-Agonists and Thyreostats in Animal Tissues by LC/MS-MS and in-line Fluorescence". 4th International Symposium on Hormone and Veterinary Drug Residue Analysis, Antwerp, Belgium, June 4-7, 2002.

SOP No: CLG-TST2.00		Page 21 of 21
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Approved by:	Date approved:
Eric Flynn	11/29/04
Bill Koscinski	11/29/04
Gina McLeroy	11/29/04
Jess Rajan	11/29/04
Charles Pixley	11/29/04
Phyllis Sparling	11/29/04

Approval signatures on file.